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ORIGINAL ARTICLE ***Reproductive endocrinology***

Ovarian reserve assessment in users of oral contraception seeking fertility advice on their reproductive lifespan

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STUDY QUESTION: To what extent does oral contraception (OC) impair ovarian reserve parameters in women who seek fertility assessment and counselling to get advice on whether their remaining reproductive lifespan is reduced?

SUMMARY ANSWER: Ovarian reserve parameters defined by anti-Müllerian hormone (AMH), antral follicle count (AFC) and ovarian volume were found to be significantly decreased by 19% (95% CI 9.1–29.3%), 18% (95% CI 11.2–24.8%) and 50% (95% CI 45.1–53.7%) among OC users compared with non-users.

WHAT IS KNOWN ALREADY: AMH and AFC have proved to be reliable predictors of ovarian ageing. In women, AMH declines with age and data suggest a relationship with remaining reproductive lifespan and age at menopause. OC may alter parameters related to ovarian reserve assessment but the extent of the reduction is uncertain.

STUDY DESIGN, SIZE, DURATION: A cross-sectional study of 887 women aged 19–46 attending the Fertility Assessment and Counselling Clinic (FACC) from 2011 to 2014 comparing ovarian reserve parameters in OC users with non-OC users.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The FAC Clinic was initiated to provide individual fertility assessment and counselling. All women were examined on a random cycle day by a fertility specialist. Consultation included; transvaginal ultrasound (AFC, ovarian volume, pathology), a full reproductive history and AMH measurement. Women were grouped into non-users and users of OC (all combinations of estrogen-progestin products and the contraceptive vaginal ring). Non-users included women with an intrauterine device (IUD) or no hormonal contraception.

MAIN RESULTS AND THE ROLE OF CHANCE: Of the 887 women, 244 (27.5%) used OC. In a linear regression analyses adjusted for age, ovarian volume was 50% lower (95% CI 45.1–53.7%), AMH was 19% lower (95% CI 9.1–29.3%), and AFC was 18% lower (95% CI 11.2–24.8%) in OC users compared with non-users. Comparison of AMH at values of <10 pmol/l OC was found to have a significant negative influence on AMH (OR 1.6, 95% CI 1.1; 2.4, $P = 0.03$). Furthermore, we found a significant decrease in antral follicles sized 5–7 mm ($P < 0.001$) and antral follicles sized 8–10 mm ($P < 0.001$) but an increase in antral follicles sized 2–4 mm ($P = 0.008$) among OC users. The two groups (OC users versus non-users) were comparable regarding age, BMI, smoking and maternal age at menopause.

LIMITATIONS, REASON FOR CAUTION: The study population comprised women attending the FAC Clinic. Recruitment was based on self-referral, which could imply a potential selection bias. Ovarian reserve was examined at a random cycle day. However, both AMH and AFC can be assessed independently of the menstrual cycle. The accuracy in predicting residual reproductive lifespan is still needed in both users and non-users of OC.

WIDER IMPLICATIONS OF THE FINDINGS: OC has a major impact on the ovarian volume, and a moderate impact on AFC and AMH with a shift towards the smaller sized antral follicle subclasses. The most evident reduction occurs in the antral follicles of 5–7 and 8–10 mm with the highest number of AMH secreting granulosa cells. It is essential to be aware of the impact of OC use on ovarian reserve parameters when guiding OC users on their fertility status and reproductive lifespan.

STUDY FUNDING/COMPETING INTEREST(S): The FAC Clinic was established in 2011 as part of the ReproHigh collaboration. This study received funding through the Capital Region Research Fund and by EU-regional funding. There are no competing interests.

TRIAL REGISTRATION NUMBER: The biobank connected to FAC Clinic is approved by the Scientific Ethical Committee (H-1-2011-081).

Key words: ovarian reserve assessment / oral contraception / AMH / ovarian volume / AFC

Introduction

The introduction of oral contraceptives (OC) in 1958 dramatically changed the way in which women and couples worldwide viewed family planning (van Heusden *et al.*, 2002). In Western countries, 50–89% of women use OC at some point in their lifetime and in Denmark 32% of fertile women are current users (Skouby, 2004; Jones *et al.*, 2012; Wilson *et al.*, 2012).

Modern women strive for higher education and career opportunities and many postpone childbearing despite the risk of low fecundity with increasing age (Schmidt *et al.*, 2012). Planning major life events such as pregnancies are essential for many women (Benzies *et al.*, 2006) and new technologies and changed legislations have extended the reproductive choices. Today, oocyte freezing and use of donor sperm in single women are possible. Oocyte freezing is widely available, and although the long-term efficiency of this procedure remains to be documented, it seems highly dependent on the ovarian age and thus oocyte quality (Rienzi *et al.*, 2012). In addition, many countries now allow single women to enter fertility treatment. As a consequence, ovarian reserve assessment is no longer just relevant for women undergoing treatment for infertility. Indeed, there has been an increased demand for ovarian reserve testing from women with no known fertility problem to obtain estimates on their remaining reproductive lifespan (Tremellen and Savulescu, 2014; Hvidman *et al.*, 2015; Seifer *et al.*, 2015).

Thus, reliable assessment of ovarian reserve is essential. Serum anti-Müllerian hormone (AMH) concentration is an indirect marker of the number of antral follicles in the ovary and thereby the ovarian reserve (La Marca *et al.*, 2010). Recent studies indicated that the ovarian reserve parameters defined by AMH, antral follicle count (AFC) and ovarian volume were lower in women using sex steroid hormones for contraception (van den Berg *et al.*, 2010; Bentzen *et al.*, 2012; Dewailly *et al.*, 2014), while others have not (Deb *et al.*, 2012). Screening of the ovarian reserve before commencement of oral contraception has recently been suggested in order to detect women with premature ovarian insufficiency (Kushnir *et al.*, 2014).

In order to be able to counsel OC users on their reproductive lifespan, we need robust studies to establish the impact of OC use on ovarian reserve parameters such as AMH and AFC.

Therefore, the aim of the present study was to analyse the impact of OC on the ovarian reserve parameters in a large cohort of users and non-users of OC seeking fertility assessment and counselling at Rigshospitalet (RH), Copenhagen.

Materials and Methods

The Fertility Assessment and Counselling Clinic

Data for this prospective cohort study were collected as part of the Fertility Assessment and Counselling Clinic (FAC Clinic) at RH, Denmark. Briefly, the FAC Clinic was established in August 2011 with the purpose of offering women and men with no known fertility problems, assessment and counselling on their present and future fertility. The clinic was open to men and women living in the Capital Region of Denmark or southern part of Sweden. The FAC Clinic was initially partly funded by the European Union (EU) Interregional projects 'Reprosund' and 'ReproHigh' from 2011 until 2014. The current funding of the FAC Clinic is provided by Rigshospitalet. Consultations at the FAC Clinic were free of charge and clients needed no referral to get an appointment. All completed a web-based baseline questionnaire (SurveyExact) before the consultation (see Supplementary data). The questionnaire included items regarding socio-demographic background, reproductive and medical history, lifestyle and behavioural exposures, such as smoking, alcohol and exercise. All women were examined by a fertility specialist, who performed a transvaginal ultrasound (AFC, ovarian volume, pathology), uptake of a full reproductive history and AMH measurement. The men had a sperm analysis performed. The concept of the FAC Clinic is described in detail in a previous paper (Hvidman *et al.*, 2015).

Assessment of hormonal contraception use

In the baseline questionnaire, the women were asked to report both the use of current and former contraceptive methods and the duration of each. The women were asked about the following contraceptives methods: (i) oral contraception with a combination of estrogen and progestin, (ii) contraceptive patches, (iii) progestin implants, (iv) contraceptive vaginal ring, (v) progestin-only products (pills), (vi) intrauterine device (IUD) with copper or levonorgestrel, (vii) i.m. depot of progestin, (viii) withdrawal, and (ix) 'safe periods'. At the consultation, the women were additionally asked to report their current contraceptive method, if any.

Assessment of ovarian reserve parameters

The number of antral follicles was counted and grouped into three predefined categories: 2–4 mm, 5–7 mm and 8–10 mm. The ovarian volume was measured by the formula for a prolate ellipsoid using the longest longitudinal (d1), anteroposterior (d2), and transversal diameters (d3): volume $= d1 \times d2 \times d3 \times \pi/6$ (Rosendahl *et al.*, 2010). Throughout the 3-year-period the same team of five doctors examined the women.

The blood test for AMH was taken at the consultation. The serum AMH concentrations were measured at the Department of Clinical Biochemistry by an enzyme-linked immunosorbent assay (ELISA) (Immunotech, Beckman Coulter Generation I, Inc., Marseilles, France). The sensitivity was 0.7 pmol/l and the intra- and inter-assay coefficients of variation were 12.3 and 14.2% (Bentzen *et al.*, 2012).

An AMH threshold value of 3, 5 and 10 pmol/l was chosen in accordance to the following: The value of 3 pmol/l was the lower limit of quantification (Beckmann Coulter Generation I). The threshold value of 5 pmol/l was the fifth percentile measured in a previous study of 1500 women in their mid-thirties conducted by the Department of Clinical Biochemistry at Rigshospitalet, Denmark (unpublished). The threshold value of 10 pmol/l was an arbitrary choice associated with the Risk Assessment Score Sheet used at the FAC Clinic (Hvidman *et al.*, 2015). The value of 10 pmol/l was equivalent to the 10th percentile among women in their early thirties in the aforementioned study of 1500 women at Rigshospitalet.

The AFC threshold values of 3, 5 and 10 were chosen based on the assumption of a high correlation and one-to-one relationship among low

numbers of AFC and AMH when using the Beckman Coulter Gen I assay in pmol/l (Bentzen et al., 2013a,b; Dewailly et al., 2014).

Assessment of covariates

Smoking, alcohol, maternal age at menopause, and prenatal exposure to maternal smoking were reported in the baseline questionnaire and were addressed again at the consultation. Gestational age at birth was only reported in the questionnaire. The women's smoking status was categorized as; non-smokers, a daily use of 1–10 cigarettes or a daily use above 10 cigarettes. Alcohol consumption was categorized as; none alcohol units per week, 1–6 alcohol units per week or more than 7 alcohol units per week. Weight, height and body mass index (BMI) were measured at the consultation.

Statistical analysis

Baseline characteristics were summarized as either mean and standard deviation (SD) of normally distributed outcomes, median and 90% population limit of non-normally distributed quantitative outcomes or number and percentage of categorical outcomes. We compared demographic characteristics, endocrine values and sonographic data between OC users and non-users by the two-sample t test and the non-parametric Mann–Whitney U test, whichever was most appropriate. Categorical variables were compared with Pearson Chi-Square or Fisher's Exact test. The association between AFC and serum AMH values was assessed by Pearson's correlation coefficient (ρ) after log transforming both outcomes to ensure an approximately normal joint distribution. To determine the age-related decline in AMH, AFC and ovarian volume logarithmic transformation were applied due to skewed distributions. The transformation implied that the estimated levels of serum-AMH and AFC were expressed as medians, and estimated differences between groups were expressed as relative (i.e. %-wise) differences. In addition, the differences in ovarian reserve parameters between users and non-users of OC were estimated in multiple linear regression analysis which included potential confounders: hormonal contraception, smoking, BMI, preterm birth, prenatal exposure to maternal smoking and maternal age at menopause. We imposed a non-inferiority assumption on the intercept of the model to compensate for the possible bias of non-randomly distributed missing data from the youngest participants with mothers experiencing normal to late onset of menopause as described in a previous paper (Bentzen et al., 2013a,b). Non-linear regression models, previously described by Hansen et al. (2008) and validated by Knowlton et al. (2014), were applied to estimate the differences in median AMH, AFC and ovarian volume with adjustment for a potentially non-linear age-related decline. The overall fit of the non-linear models was compared with the corresponding linear fits. We used bootstrapping to ensure that P-values and 95% confidence intervals obtained from the non-linear model were valid (Davison and Hinkley, 1997). Multiple logistic regression with adjustment for age was applied to test whether the risk of having an AMH or AFC $<$ 3, 5 or 10 differed between users and non-users of OC. Duration of hormonal contraception was found to be highly collinear with age. Thus to assess a possible effect of duration on AMH, AFC and ovarian volume in OC users, these were transformed to age-adjusted Z-scores prior to analysis. We used the group of non-users as reference for computing the Z-scores. Descriptive statistics were made with the statistical software SPSS (version 19, Chicago, USA) and Microsoft Office Excel 2010; regression analyses were performed with R (version 2.13.2, Vienna, Austria).

Ethical approval

All participants gave written informed consent according to the Declaration of Helsinki for Medical Research involving Human Subjects. The establishment of a biobank was approved by the Scientific Ethical Committee of the Capital Region of Denmark (journal number: H-1-2011-081).

Results

Study population

A total of 971 women aged 19–46 were examined at the FAC Clinic. In our analyses, we excluded 62 women. Reasons for exclusion were: (i) pregnancy discovered at the consultation ($n = 9$), (ii) present fertility treatment ($n = 1$), (iii) no available baseline questionnaire ($n = 29$), (iv) failed AMH analysis ($n = 3$) or (v) no-show at the consultation ($n = 20$). In total, 909 women were eligible for the present study (Fig. 1).

The study population was homogeneous in relation to education level, ethnicity (primarily Caucasians) and lifestyle factors. Demographic characteristics in OC users versus non-users are shown in Table I.

The women's contraceptive method was categorized into five groups as illustrated in Fig. 1: (i) OC's (all combinations of ethinyl estradiol and progestin) ($n = 225$), (ii) IUD with levonorgestrel ($n = 12$), (iii) progestin-only products incl. implants ($n = 22$), (iv) contraceptive vaginal ring ($n = 19$) and (v) no hormonal contraception ($n = 631$).

As visualized in Fig. 1, these groups were condensed into the following two groups for analytic purposes: (i) OC users ($n = 244$) (all ethinyl estradiol and progestin oral products or vaginal ring) and (ii) non-users ($n = 643$) (IUDs or no hormonal contraception). In our analysis we excluded the women using progestin-only pills ($n = 21$) and implants ($n = 1$). Therefore the final study population comprised 887 women. The 244 users of OC were significantly younger than non-users with a mean age of 31.5 (SD 4.3) versus 34.1 (SD 4.3) years ($P < 0.001$). Overall, and when stratifying by age groups, there was no difference between the two groups in relation to bodyweight, BMI, smoking habits, gestational age at birth, prenatal exposure to maternal smoking or maternal age at menopause.

Motives for attending the FAC Clinic and current pregnancy wish

The women's main motives for attending the clinic were (i) knowledge on how long they can postpone childbearing (70%), (ii) concern about their fecundity (63%), (iii) information on how to preserve/optimize their chances of having children (48%) and (iv) the woman was presently trying to conceive and wanted an estimate of her pregnancy chances (28%).

In all 49% (433/887) had a pregnancy wish at the time of the consultation, yet only 23% (200/887) were actually trying to achieve a pregnancy. A total of 218/643 (34%) among the non-OC users did not use any kind of contraception.

Menstrual cycle length among the non-OC users

The menstrual cycle pattern was listed for 630 of the 643 non-OC users and was distributed as follows: (i) Polymenorrhea (0–20 days): 4/630 (none with an IUD with levonorgestrel), (ii) regular cycle (21–35 days): 580/630 (five women had an IUD with levonorgestrel), (iii) Oligomenorrhea (36–180 days): 40/630 (three women had an IUD with levonorgestrel), or (iv) Amenorrhoea (longer than 180 days): 6/630 (four women had an IUD with levonorgestrel).

Correlation between AMH and AFC

The overall correlation between log-AMH and log-AFC was 0.76 (95% CI 0.73; 0.78) (Fig. 2). The correlation was stronger in the non-users

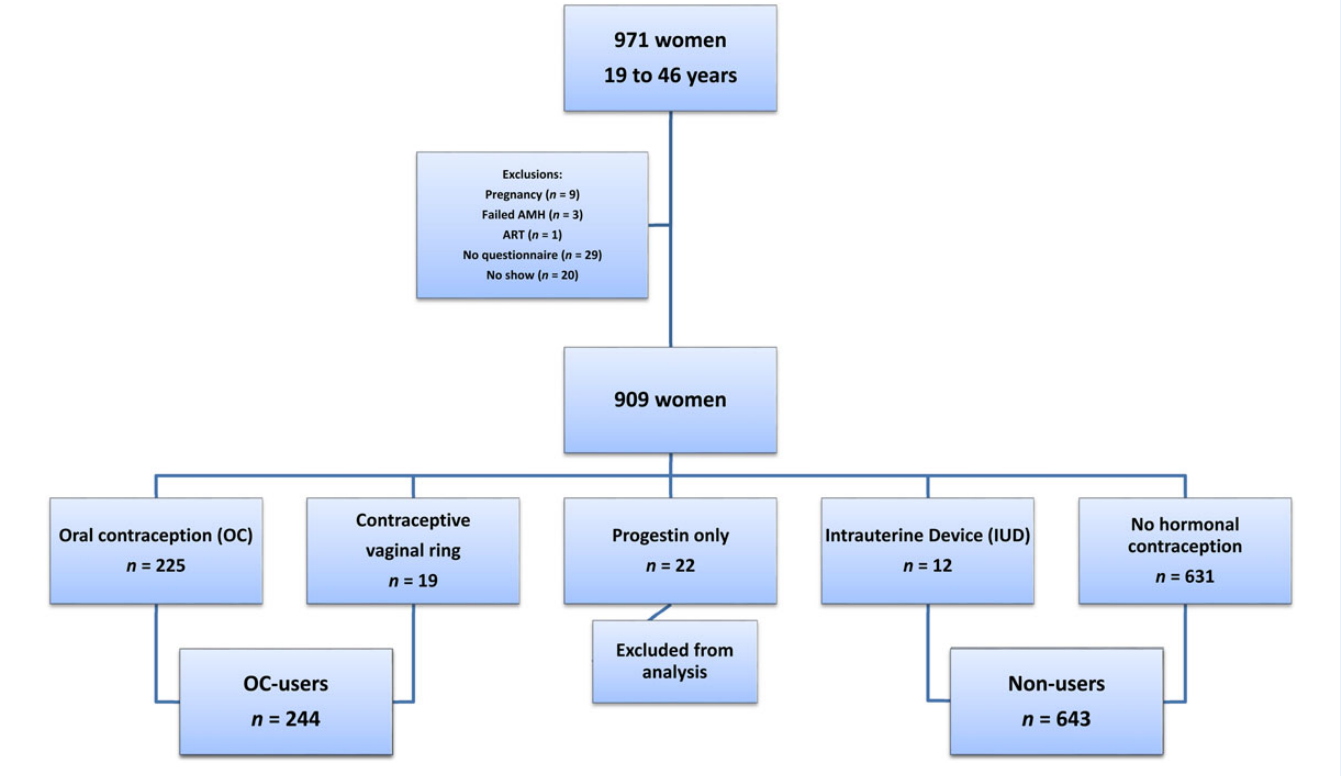

Figure 1 Diagram of the initial cohort of 971 women and the distribution of included OC users and non-users.

(ρ = 0.78, 95% CI 0.75; 0.81) compared with the OC group (ρ = 0.69, 95% CI 0.61; 0.75).

Anti-Müllerian hormone

Table II provides a comparison of AMH between OC users and non-users. Overall, no significant difference was found in AMH between the two groups. When adjusting for age by linear regression analysis, we found an overall reduction in AMH of 19% compared with non-users ($P < 0.001$, 95% CI 9.1%; 29.3%). In the multiple regression analysis adjusted for smoking, BMI, preterm birth, prenatal exposure to maternal smoking and maternal age of menopause, we found a similar reduction in AMH of 20% in the OC group ($P < 0.001$, 95% CI 8.4%; 30.3%). Hansen's power model was applied when a non-linear effect of aging was assumed (Hansen *et al.*, 2008). The difference between users and non-users of OC was similarly estimated to be 19% (95% CI 9.2–28.1). As shown in Fig. 3a, the suppressive effect of OC on AMH values seemed to be most pronounced among the young and old women, but this may be a chance finding as no significant interaction was found between the effect of age and use of OC ($P = 0.37$).

The distribution of women with an AMH below 3, 5 and 10 pmol/l in the two groups is illustrated in Table III. We found significantly more women with an AMH < 5 pmol/l in the young age group from 19 to 29.9 years among OC users than non-users ($P = 0.044$). Yet, only for AMH < 10 pmol/l the negative influence of OC was significant (OR 1.6, 95% CI 1.1;2.4, $P = 0.03$) based on a logistic regression adjusted for age.

Antral follicle count

Data on AFC are presented in Table II. The linear regression analysis adjusted for age and the multiple regression analysis adjusted for smoking, BMI, preterm birth, prenatal exposure to maternal smoking and maternal age of menopause showed a decline in AFC of 18% ($P < 0.001$, 95% CI 11.2%; 24.8%) and 17% ($P < 0.001$, 95% CI 8.7%; 24.6%), respectively. Similarly, the estimated reduction was 18% (95% CI 11.2–24.8%) based on Hansen's power model assuming a non-linear effect of aging, the diminishing effect of OC on AFC appeared to be most evident in the younger and older age groups as illustrated in Fig. 3b, although no significant interaction between the effect of OC and age was found ($P = 0.06$).

Table III additionally displays the distribution of women with an AFC below 3, 5 and 10 in OC users and non-users. The negative influence of OC on AFC was significant in all three groups: AFC ≤ 3 (OR 3.8, 95% CI 1.1;13.1, $P = 0.03$), AFC < 5 (OR 4.4, 95% CI 1.8;10.5, $P = 0.001$) and AFC < 10 (OR 2.4, 95% CI 1.6;3.6, $P = 0.0001$) based on a logistic regression adjusted for age.

Overall, we found a decreasing proportion of the small AFC (2–4 mm) with increasing age in both groups (Table II and Table IV). Among the non-users, the proportion dropped from 70% (age group 19–29.9) to 54% (age group 40–46) ($P < 0.001$). Among OC users, the proportion declined from 84% (age group 19–29.9) to 69% (age group 35–39.9) ($P < 0.001$). The group of OC users from 40 to 46 years was excluded from the analysis due to low numbers ($n = 2$). In both non-users and OC users an increased proportion of antral follicles sized 5–7 mm and 8–10 mm were observed with increasing age. The

Table I Demographic characteristics of hormonal contraceptive users ($n = 244$) and non-users ($n = 643$).

	Oral contraceptive users (*n*)					Non-users (*n*)					
Age groups	**19–29.9**	**30–34.9**	**35–39.9**	**≥40**	**Total**	**19–29.9**	**30–34.9**	**35–39.9**	**≥40**	**Total**	***P*-values[†]**
Number	87	89	66	2	244	101	224	258	60	643	
Age, mean (SD)	27.0 ± 2.3	32.0 ± 1.5	36.6 ± 1.4	41 ± 1.4	31.5 ± 4.3	27.3 ± 2.0	32.2 ± 1.5	36.8 ± 1.4	41.3 ± 1.3	34.1 ± 4.3	0.001*[,a]
Clinical characteristics											
Body weight, kg, mean, SD	62.5 ± 10.4	63.9 ± 11.4	64.5 ± 7.9	74.5 ± 14.8	63.6 ± 10.2	63.4 ± 10.4	65.1 ± 12.4	66.3 ± 12.1	67.4 ± 12.4	65.5 ± 12.0	0.750[a]
BMI, mean, SD	22.0 ± 3.3	22.8 ± 3.7	22.5 ± 2.9	26.4 ± 1.9	22.5 ± 3.4	22.4 ± 3.4	22.8 ± 3.9	23.2 ± 4.0	23.7 ± 3.9	23.0 ± 3.9	0.100[a]
Cigarettes per day, *n*, %											
None, not daily	68 (78.2)	77 (86.5)	59 (89.4)	2 (100)	206 (84.8)	86 (85.1)	197 (87.9)	215 (83.3)	52 (86.7)	550 (85.5)	0.826
1–10 cigarettes	16 (18.4)	9 (10.2)	5 (7.6)	0	30 (12.3)	13 (12.9)	22 (9.8)	28 (10.9)	5 (8.3)	68 (10.6)	
>10 cigarettes	3 (3.4)	2 (2.3)	2 (3.0)	0	7 (2.9)	2 (2.0)	5 (2.2)	15 (5.8)	3 (5.0)	25 (3.9)	
Gestational age at birth, weeks, *n*, %											
<32 weeks	2 (2.3)	1 (1.1)	0	0	3 (1.2)	0	3 (1.3)	2 (0.8)	0	5 (0.8)	0.121[b]
32–37 weeks	4 (4.6)	1 (1.1)	1 (1.5)	0	6 (2.5)	4 (5.0)	14 (6.3)	18 (7.0)	4 (6.7)	40 (3.2)	
≥38 weeks	80 (91.9)	87 (97.7)	65 (98.5)	2 (100)	234 (95.9)	97 (95.0)	205 (91.5)	237 (91.9)	56 (93.3)	595 (92.5)	
Missing	1 (1.1)	0	0	0	1 (0.4)	0	2 (0.9)	1 (0.4)	0	3 (0.5)	
Prenatal exposure to maternal smoking, *n*, %											
Yes	29 (33.3)	31 (34.8)	14 (21.2)	1 (50.0)	75 (30.7)	34 (33.7)	73 (32.6)	83 (32.2)	13 (21.7)	203 (31.6)	0.548[b]
No	56 (64.4)	47 (52.8)	45 (68.2)	1 (50.0)	149 (61.1)	62 (61.4)	138 (61.6)	153 (59.3)	44 (73.3)	397 (61.7)	
Don't know	2 (2.2)	11 (12.4)	7 (10.6)	0	20 (8.2)	5 (5.0)	13 (5.8)	22 (8.6)	3 (5.0)	43 (6.7)	
Maternal age at menopause, years, *n*, %											
<45 years	7 (8.0)	8 (9.0)	6 (9.1)	0	21 (8.6)	4 (4.0)	15 (6.7)	13 (5.0)	4 (6.7)	36 (5.6)	0.205[b]
45–50	32 (36.8)	28 (31.5)	17 (25.8)	1 (50.0)	78 (32.0)	34 (33.7)	75 (33.5)	86 (33.3)	16 (26.7)	211 (32.8)	
>50	32 (36.8)	35 (39.3)	24 (36.4)	0	91 (37.3)	43 (42.6)	100 (44.6)	100 (38.8)	22 (36.7)	265 (41.2)	
Don't know	16 (18.4)	18 (20.2)	19 (28.8)	0 (50.0)	54 (22.1)	20 (19.8)	34 (15.2)	59 (22.9)	18 (30.0)	131 (20.4)	

[†]*P*-values indicates the difference between the hormonal contraceptive users and non-users.
*Significant $P < 0.05$.
[a]Mann–Whitney *U* test, Kruskall–Wallis.
[b]Pearson χ^2 test.

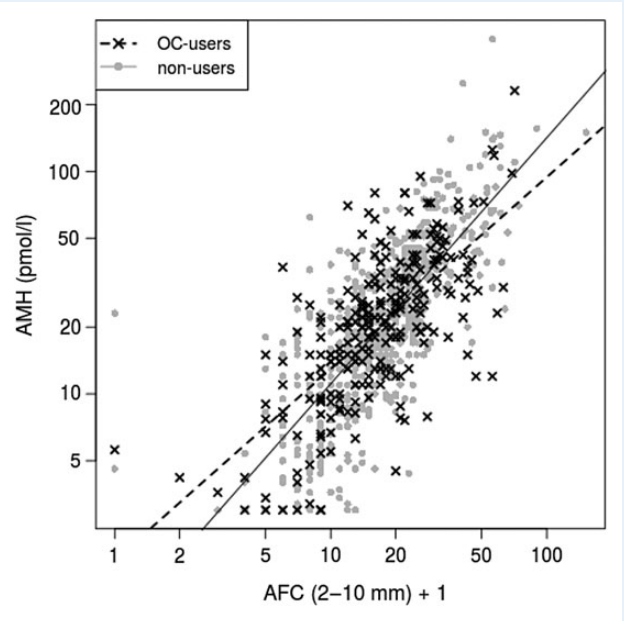

Figure 2 Correlation analysis of AMH and AFC. The correlation between log-AMH and log-AFC visualized by a scatter plot in a logarithmic scale.

proportion of antral follicles sized 5–7 mm rose from 24% (age group 19–29.9) to 29% (age group 40–46) ($P < 0.009$) and the proportion of the large antral follicles sized 8–10 mm from 5% (age group 19–29.9) to 11% (age group 35–39.9) ($P < 0.001$) among non-users. In the OC users, the proportion of antral follicles sized 5–7 mm increased from 13% (age group 19–29.9) to 25% (age group 35–39.9) ($P < 0.001$) and the antral follicles sized 8–10 from 3% (age group 19–29.9) to 6% (age group 35–39.9) ($P = 0.07$). Furthermore, we found a significant decrease in antral follicles sized 5–7 mm ($P < 0.001$) and antral follicles sized 8–10 mm ($P < 0.001$) and an increase in antral follicles sized 2–4 mm ($P = 0.008$) among OC users compared with non-users (Table II). The differences in AFC according to size between non-users and OC users stratified by age groups are displayed in Table IV.

Ovarian volume

The ovarian volume was markedly reduced by 49.6% when assessed by the age-adjusted linear regression analysis in OC users compared with non-users ($P < 0.001$, 95% CI 45.1%; 53.7%). The multiple regression analysis adjusted for smoking, BMI, preterm birth, prenatal exposure to maternal smoking, and maternal age at menopause found a reduction of 48.6% ($P < 0.001$, 95% CI 43.2%; 53.4%). Hansen's power model for non-linear age-decline illustrated in Fig. 3c found a reduction of 49.6% ($P < 0.001$, 95% CI 45.1%; 53.7%). Stratified by age groups, the significant reduction in the right ovarian volume ranged from 30% (40–46 years) to 50% (30–34.9 years) in OC users. The reduction in left ovarian volume was likewise significant and ranged from 37% (40–46 years) to 53% (19–29.9 years) (Table II).

Covariate analysis

To determine the influence of the covariates on the ovarian reserve parameters AMH, AFC and ovarian volume we performed a multiple regression analysis regarding hormonal contraception, smoking, BMI, preterm birth, prenatal exposure to maternal smoking, and maternal age at menopause. In relation to AMH we found a significant influence of maternal age at menopause ($P < 0.005$). The rates of decay of AMH per year of aging increased with a decreasing age of maternal menopause (8.7% maternal menopause before 45 years versus 6.6% maternal menopause above 50 years). We found a comparable result for AFC ($P = 0.006$) with a decay of 6.3 versus 5.1% per year of aging in the same maternal menopausal groups. None of the remaining covariates had a significant impact on AMH and AFC. Furthermore, no significant association of the covariates was found on the ovarian volume.

Duration of hormonal contraception

The duration of current hormonal contraception was listed for 64% (155/244). The median duration in the OC group was 12 years (90% population limit: 4; 18.4 years). We found no significant effect of duration of hormonal contraception on AMH ($P = 0.99$), AFC ($P = 0.44$) or ovarian volume ($P = 0.08$) after adjusting for expected age-related decline but a trend towards smaller ovaries with longer duration of use among current OC users.

Discussion

We found significant reductions in the ovarian reserve parameters defined by AMH (20% decrease) and AFC (18% decrease) and a more pronounced reduction by 50% of the ovarian volume among OC users compared with non-users in a cohort of women seeking fertility assessment and counselling.

Moreover, a critically low value of AMH < 5 pmol/l was seen in 5% of OC users younger than 30 years of age compared with none among the non-OC users of the same age although this could be a chance finding. Yet, the negative influence of OC among women with an AMH < 10 pmol/l was significant. Similarly, OC had a significant negative influence on AFC in users compared with non-users when adjusted for age.

To our knowledge, this is the largest study investigating the impact of OC on all ovarian reserve parameters (AMH, AFC and ovarian volume) to date. Our results are in accordance with earlier studies (Christensen *et al.*, 1997; Bentzen *et al.*, 2012; Dolleman *et al.*, 2013a,b; Kallio *et al.*, 2013; Johnson *et al.*, 2014) and could have implications for the interpretation of ovarian reserve assessment in OC users. Our findings underline the risk of falsely identifying a low ovarian reserve in users of OC. Additionally, OC use can mask premature ovarian insufficiency, which will affect 1% of the fertile women. Ovarian reserve assessment should therefore be performed with caution in OC users as they may have altered reproductive markers. As discussed by Hvidman *et al.* such 'false' identification of low ovarian reserve could have major consequences for the women in terms of anxiety and the risk of overtreatment (Hvidman *et al.*, 2015).

We detected a significant decrease of the small AFC sized 2–4 mm with age. Interestingly, we observed a similar shift towards the smaller AFC subclasses in OC users. OC affects the gonadotrophin secretion through direct pituitary suppression (van Heusden *et al.*, 2002) and the extent depends on the type and dose of steroids used, the administration regimen, user compliance, and the responsiveness of the woman taking the hormones (Baerwald and Pierson, 2004). The estrogen component in combination with the effect of peripheral progestin inhibits the

Table II Endocrine parameters and sonographic characteristics of hormonal contraceptive users ($n = 244$) and non-users ($n = 643$).

	Oral contraceptive users (*n*)					Non-users (*n*)					
Age groups	**19–29.9**	**30–34.9**	**35–39.9**	**≥40**	**Total**	**19–29.9**	**30–34.9**	**35–39.9**	**≥40**	**Total**	***P*-values[†]**
Number	87	89	66	2	244	101	224	258	60	643	
Endocrine parameters (serum)											
AMH, pmol/l, median (90% population limit)	25 (9.1;55.2)	21 (7.9;61.0)	15.5 (4.6;47.2)	3.8 (3.4;4.2)	21 (7.2;52.0)	33 (12.2;85.8)	24 (9.6;56.0)	17 (4.9;48.1)	11 (3.0;31.9)	22.0 (5.9;53.8)	0.519[a]
Sonographic characteristics											
AFC, median (90% population limit)	20 (8.0;43.2)	15 (8.0;30.0)	12 (5.0;25.9)	3.5 (3.0;4.0)	15.5 (7.0;34.5)	23 (13.0;51.2)	19 (10.0;37.0)	14 (6.0;28.1)	9.5 (4.0;19.8)	17 (7.0;34.5)	0.258[a]
AFC 2–4 mm	16.5 (5.7;33.6)	13 (5.9;25.1)	8.5 (2;20.0)	1.5 (1.0;2.0)	12 (4.0;28.0)	15 (7.0;34.0)	13 (5.0;25.5)	9 (3.0;19.1)	4 (1.0;15.0)	11 (3.0;24.7)	0.008[a,*]
AFC 5–7 mm	0 (0;13.0)	1 (0;8.1)	2 (0;8.3)	0.5 (0;1.0)	1 (0;9.0)	5 (0;11.8)	4 (0;10.0)	3.5 (0;10.0)	2 (0;8.0)	4 (4.0;10.0)	0.001[a,*]
AFC 8–10 mm	0 (0;2.0)	0 (0;1.1)	0 (0;3.0)	1.5 (0;3.0)	0 (0;2.0)	0 (0;4.0)	1 (0;4.0)	1 (0;4.0)	1 (0;4.0)	1 (0;4.0)	0.001[a,*]
AFC total right ovary	11 (4.0;22.6)	8 (3.9;16.0)	6.5 (2.0;13.0)	3 (3.0;3.0)	8 (3.0;18.0)	13 (7.0;27.4)	10 (5.0;22.0)	7.5 (3.0;15.0)	5 (2.0;11.0)	9 (3.0;19.0)	0.525[a]
AFC total left ovary	9 (3.0;20.0)	7 (3.0;15.0)	6 (2.0;14.3)	0.5 (0;1.0)	7 (3.0;16.0)	11 (5.0;25.0)	9 (3.0;16.5)	7 (3.0;14.0)	4 (2.0;10.0)	8 (3.0;16.0)	0.286[a]
Right Ovarian volume, ml, median (90% population limit)	3.3 (1.1;6.2)	2.9 (1.1;7.5)	3.1 (1.2;6.9)	3.2 (0.6;5.69)	3.1 (1.2;7.1)	6.4 (2.9;11.7)	5.8 (2.7;11.9)	5.9 (2.9;11.4)	4.6 (1.7;8.6)	5.8 (2.6;11.5)	0.001[a,*]
Left Ovarian volume, ml, median (90% population limit)	2.8 (1.1;6.2)	2.9 (1.1;7.3)	2.5 (0.7;7.5)	2.4 (1.4;3.35)	2.7 (1.1;7.0)	5.9 (2.6;12.3)	5.4 (2.6;11.6)	4.9 (2.2;10.8)	3.8 (1.4;8.7)	5.2 (2.3;11.0)	0.001[a,*]

AMH, anti-Müllerian hormone; AFC, antral follicle count.

[†]*P*-values indicate the difference between the hormonal contraceptive users and non-users.

*Significant $P < 0.05$.

aMann–Whitney *U* test, Kruskall–Wallis.

bPearson χ^2 test.

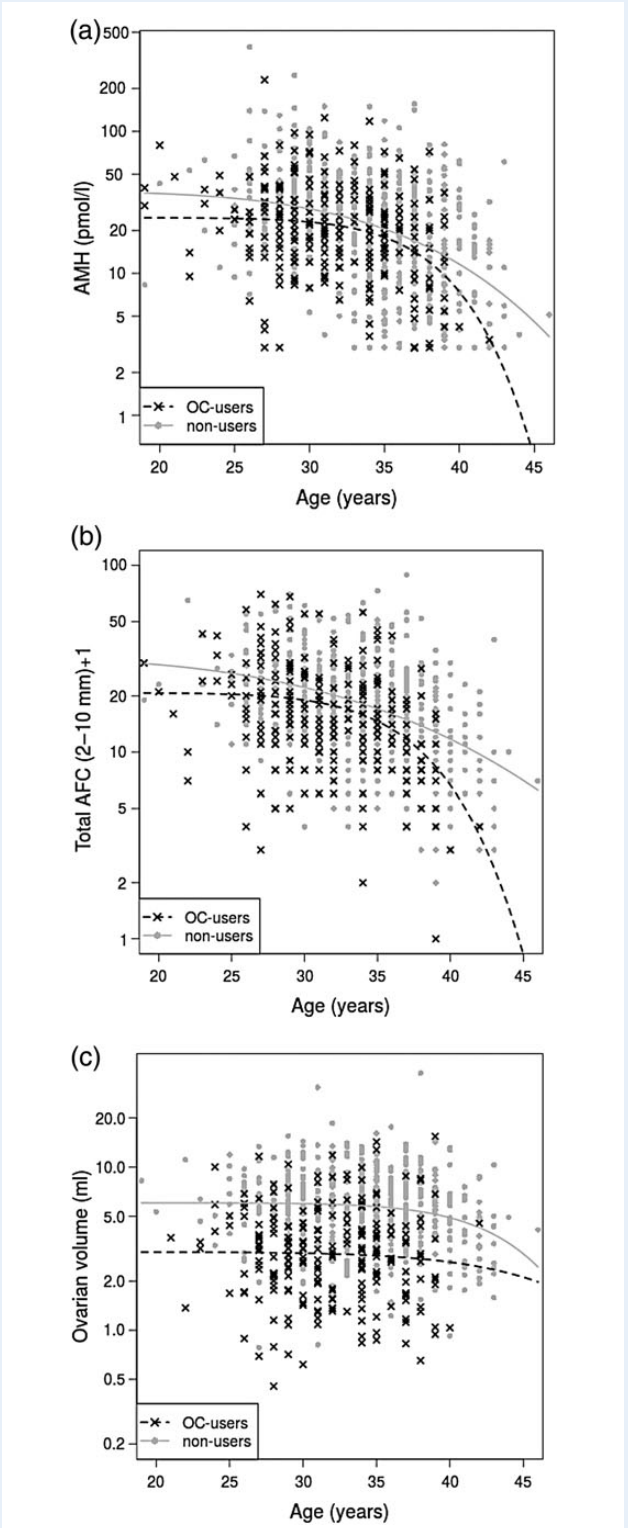

Figure 3 Relation between chronological age and ovarian reserve parameters among hormonal contraceptive users ($n = 244$) compared with non-users ($n = 643$). (**a**) Hansen's power model and the non-linear association of age on AMH. (**b**) Hansen's power model and the non-linear association of age on AFC. (**c**) Hansen's power model and the non-linear association of age on ovarian volume. Data are displayed in a logarithmic scale.

Table III The distribution of low AMH and AFC values in hormonal contraceptive users ($n = 244$) and non-users ($n = 643$).

	Oral contraceptive users (*n*)					Non-users (*n*)						
Age groups	**19–29.9**	**30–34.9**	**35–39.9**	**≥40**	**Total**	**19–29.9**	**30–34.9**	**35–39.9**	**≥40**	**Total**	***P*-values†**	**95% CI†**
Number	87	89	66	2	244	101	224	258	60	643		
AMH values, *n* (%)												
AMH ≤ 3	2 (2.3)	0	4 (6.1)	0	6 (2.5)	0	2 (0.9)	9 (3.5)	11 (18.3)	22 (3.4)	0.28[a]	0.6;4.7
AMH < 5	4 (4.6)*	2 (2.2)	7 (10.6)	2 (100)	15 (6.1)	0*	6 (2.7)	26 (10.1)	15 (25.0)	47 (7.3)	0.11[a]	0.3;1.1
AMH < 10	11 (12.6)	15 (16.9)	18 (27.3)	2 (100)	46 (18.9)	6 (5.9)	24 (10.7)	69 (26.7)	26 (43.3)	125 (19.4)	0.03[a,**]	1.04;2.4
AFC values, *n* (%)												
AFC ≤ 3	1 (1.1)	1 (1.1)	2 (3.0)	1 (50.0)	5 (2.1)	0	0	5 (1.9)	3 (5.0)	8 (1.2)	0.03[a,**]	1.1;13.1
AFC < 5	2 (2.3)	2 (2.2)	5 (7.6)	1 (50.0)***	10 (4.1)	0	2 (0.9)	7 (2.7)	7 (11.7)***	16 (2.5)	0.001[a,**]	1.8;10.5
AFC < 10	12 (13.8)***	15 (6.9)	24 (36.4)***	0	51 (20.1)	3 (3.0)***	21 (9.4)	62 (24.0)***	30 (50.0)	116 (18.0)	0.0001[a,**]	1.6;3.6

AMH = anti-Müllerian hormone, values are pmol/l.
†*P*-values and 95% CI indicate the difference between the hormonal contraceptive users and non-users.
[a]Logistic regression adjusted for age.
*Significant difference of low AMH < 5 pmol/l between oral contraceptive users compared with non-users ($P = 0.044$).
**Significant $P < 0.05$.
***Significant difference of low AFC between oral contraceptive users compared with non-users ($P < 0.05$).

Table IV The difference in AFC among hormonal contraceptive users ($n = 244$) compared with non-users ($n = 643$).

	Non-users[a]				Oral contraceptive users[a]				Difference in AFC	P-values[†,b]
Age groups	19–29.9	30–34.9	35–39.9	≥40	19–29.9	30–34.9	35–39.9	≥40		
Number	101	224	258	60	87	89	66	2		
AFC group										
Total 2–10 mm	26.7 (24.0;29.4)	21.8 (20.1;23.7)	16.5 (15.2;17.9)	10.9 (9.2;12.7)	23.1 (20.2;26.1)	17.8 (15.8;20.1)	14.2 (12.1;16.6)	NA	−3.3 (−5.2;−1.5)	0.001*
2–4 mm	19.0 (16.6;21.5)	14.9 (13.7;16.2)	10.2 (9.3;11.1)	6.3 (4.9;7.8)	19.0 (16.4;21.7)	14.8 (12.8;17.0)	9.7 (8.1;11.4)	NA	−0.2 (−1.7;1.3)	0.7805
5–7 mm	6.2 (5.0;7.5)	5.2 (4.4;6.2)	5.0 (4.2;5.8)	3.2 (2.4;4.2)	3.4 (2.3;4.8)	2.7 (2.0;3.5)	3.8 (2.7;5.1)	NA	−2.2 (−3.0;−1.3)	0.001*
8–10 mm	1.2 (0.7;1.7)	1.6 (1.2;2.1)	1.4 (1.2;1.7)	1.4 (1.0;1.8)	0.7 (0.3;1.2)	0.4 (0.2;0.7)	0.7 (0.4–1.1)	NA	−0.8 (−1.2;−0.5)	0.001*

Values are mean counts with 95% confidence intervals.
The age group 40–46 of hormonal contraceptive users only included two patients, why this group was excluded from the analysis.
[†]P-values indicate the difference between the hormonal contraceptive users and non-users.
*Significant $P < 0.05$.
[a]To compute the 95% confidence intervals 10.000 bootstrap samples were used.
[b]Non-parametric permutation two-way ANOVA adjusting for age.

production of FSH and hence the FSH-dependent follicle growth in OC users (van Heusden and Fauser, 1999). As described by Dolleman *et al.*, FSH is an important factor for the pre-antral and early antral follicles that produces AMH (Dolleman *et al.*, 2013a,b). The contribution of AMH by the pre-antral follicles is limited as the number of granulosa cells is much smaller (Jeppesen *et al.*, 2013). A recent study showed that the antral follicles sized 5–8 mm contribute the most to the concentration of circulating AMH (~60% of serum AMH), 20–25% by 2.1–5 mm follicles and 15–20% by >8 mm follicles (Jeppesen *et al.*, 2013). This underlines that AMH reflects the number of growing follicles and is only a proxy for the number of primordial follicles. The strong positive relationship between serum AMH and AFC, regardless of use of OC, is now well established (Andersen *et al.*, 2010; Kristensen *et al.*, 2012). The decreased overall number of antral follicles, the shift towards the smaller subclasses, the induced suppression of FSH and the known association between AFC and AMH related to lower number of AMH-producing granulosa cells, could in combination explain the diminished AMH values in OC users compared with non-users.

The abovementioned changes in the antral follicle subclasses could also explain the pronounced reduction of ovarian volume. This is in accordance to earlier studies, although the suppression is highly individual in women and possibly related to the type of pharmaceutical oral contraception used (Christensen *et al.*, 1997; ESHRE, 2001; Deb *et al.*, 2012). The marked volume reduction could be attributed to several factors. One is that the number of larger antral follicles was reduced; the second could be that no dominant, pre-ovulatory follicles or corpus luteum were present due to the suppression of ovulation in OC users. However, other mechanisms may also play a part. Circulating androgens are markedly reduced by OC. Hence, not only the activity but also the size of the androgen secreting stromal compartment of the ovaries may be reduced in OC users (Jones, 1995). Although the age-related reduction in ovarian volume in accordance to follicle depletion is well-known it is important to emphasize that the measurement of ovarian size is an inferior marker of ovarian reserve compared with AMH and AFC (Wallace and Kelsey, 2004; Bentzen *et al.*, 2013a,b).

In relation to fertility assessment and counselling on reproductive life span the clinical significance and possible consequence of a 20% reduction in AMH and AFC in a counselling situation cannot be quantified at the present. It is important to emphasize that a consultation at the FAC Clinic consists of several components apart from AMH and AFC measurement. The women are being evaluated by a thorough questionnaire, gynaecological and reproductive history, maternal dispositions, lifestyle factors and vaginal ultrasound looking for pathology. The use of AMH and AFC is primarily a quantitative measurement and should not stand alone. At present an ongoing 2-year follow-up is proceeding, but longer follow-up studies are needed to evaluate the clinical impact.

The suppressive effect of hormonal contraception is believed to be reversible within 3–6 months (van Heusden *et al.*, 2002; van den Berg *et al.*, 2010). In a cohort study of 3727 women aged 18–40 years they were not able to detect a deleterious effect on fecundability after cessation of long-term use of oral contraception, but merely a short-term delay compared with barrier methods (Mikkelsen *et al.*, 2013). Earlier studies even advocate for beneficial effects of long-term OC use in relation to an inhibition of the follicle depletion and postponement of natural menopause (Gold *et al.*, 2001; Palmer *et al.*, 2003). Recent research has verified that the velocity of the apoptosis of primordial follicles, the diminishing oocyte quality with age and the timing of natural menopause is independent of

hormonal contraceptive use (Broekmans *et al.*, 2004, 2009; Richardson *et al.*, 2014). It was recently suggested to examine the ovarian reserve parameters prior to commencement of OC, as OC can conceal premature ovarian insufficiency (Kushnir *et al.*, 2014).

The proportion of OC users in our cohort (28%) correlates with OC use in the background population of 32% (Wilson *et al.*, 2012). Furthermore, recommendations on preferred type of hormonal contraception were recently changed in women below 35 years to a combined low-risk pill with a second-generation progestin and the lowest compliable dose of ethinyl estradiol (Lidegaard, 2014). Today, two-thirds of Danish women use first or second-generation progestin with 30–40 μg of ethinyl estradiol compared with one-third in 2010 (Register of Medicinal Product Statistics, Denmark). Thus, we assume that the majority of women used quite similar OC preparations of first or second generation OC. Indications for OC use were not registered in the questionnaire as the far majority of the women started OC in their mid-to-late teens, which implied a risk for recall bias. Another possible limitation to our study is the retrospectively reported duration of OC use. Type and dose were not reported. However, earlier studies could not detect a dose–response relationship (Bentzen *et al.*, 2012; Dolleman *et al.*, 2013a,b). Neither did we register the prevalence of PCOS. The prevalence of 'true' PCOS defined by the Rotterdam Criteria is presumably over diagnosed among young women (Kristensen *et al.*, 2010; Lauritsen *et al.*, 2014) and women's androgen status was not measured. Hence, self-reported PCOS was not considered to be reliable. During recent years it has been well established that AMH may be a useful indicator of the time of menopause (Broer *et al.*, 2011; Dolleman *et al.*, 2013a,b; Dewailly *et al.*, 2014; Ramezani Tehrani *et al.*, 2014). As mentioned; genetic dispositions, environmental and lifestyle factors such as; smoking, BMI, and use of alcohol may influence menopausal timing as well (Broekmans *et al.*, 2009; Voorhuis *et al.*, 2010; Richardson *et al.*, 2014). Nevertheless, we did not find an interaction between the aforementioned lifestyle factors, preterm birth or prenatal exposure to maternal smoking in relation to AMH and AFC.

While AMH may predict age of menopause and remaining reproductive lifespan only very limited data suggest that AMH levels is related to the present fecundability after natural conceptions, although a prospective study of women in their thirties found a significantly reduced fecundability in those with a low AMH (Steiner *et al.*, 2011). A recent Danish cohort study of young women in their early twenties found no association between lower AMH levels and fecundability, which illustrates the wide range of AMH among women with a normal fertility potential (Hagen *et al.*, 2012).

A possible limitation of the study, but also strength in terms of representativity, may be the measurement of AFC and ovarian volume which could be influenced by the inter-individual variability between the five consultants. To ensure consistency the consultant team consisted of fertility experts and remained unchanged throughout the study period. In relation to measurements of AMH, a recent review stated that fluctuations of AMH in the menstrual cycle appear to be random and minor, thus permitting AMH measurement independently of the cycle phase (Dewailly *et al.*, 2014). Furthermore, the fluctuations of AMH are randomly distributed during menstrual cycle which contradicts the necessity of a fixed cycle day (Dewailly *et al.*, 2014). AFC and ovarian volume is believed to be optimally measured between cycle day 2–5 (Iliodromiti *et al.*, 2014). Yet, in our study AFC was measured at a random cycle day and our results showed the usual high correlation between log-AMH and log-AFC ($\rho = 0.76$, 95% CI 0.73–0.78). Additionally, the age-related figures in terms of AFC are almost identical to our earlier study where all had AFC measurements done at cycle day 2–5 (Bentzen *et al.*, 2012). Therefore we believe that AFC can be measured on a random cycle day. This argument is supported by two recent studies of 256 and 34 women (Kristensen *et al.*, 2012; Deb *et al.*, 2013). It could also be argued that AMH and AFC seem to be influenced similarly by OC treatment, and either of these two parameters could thus be used, saving time and resources.

Another possible limitation could be the AMH assay Beckman Coulter Gen I in terms of sensitivity compared with Gen II (Gen I: 0.7 pmol/l versus Gen II: 0.17 pmol/l), storage and handling conditions (Dewailly *et al.*, 2014). However, a recent study described that between-sample variability without regard to menstrual cycle as well as within-sample variation appeared to be higher using the Gen II AMH assay than with Gen I (Rustamov *et al.*, 2014). To prevent biased results in terms of higher AMH values when changing to Gen II (Broer *et al.*, 2014), we consistently used Gen I throughout the study period.

There is an increasing demand for fertility assessment and counselling (Kushnir *et al.*, 2014; Tremellen and Savulescu, 2014; Hvidman *et al.*, 2015; Seifer *et al.*, 2015). Women and well-educated women in particular, postpone their first pregnancy. As such, maternal age at first birth has increased all over Europe over the past four decades. Postponed childbearing implies a higher rate of involuntary childlessness, smaller families than desired and declining fertility rates (ESHRE, 2010; Schmidt *et al.*, 2012). The recruitment was based on self-referral and the majority of women were well-educated, which could imply a potential selection bias. Nevertheless, there seems to be a need for individual assessment and knowledge on the impact of OC, since almost one in three women who had an appointment in the FAC clinic used OC at the time of the consultation, and the majority had used OC for several years (mean of 12 years).

In correspondence with a recent Danish cohort study on health care workers, we found a significant negative correlation between the AMH level in women and their mothers' age at menopause (Bentzen *et al.*, 2013a,b). This finding corroborates a genetic component in the reproductive lifespan of a woman. Thus, in addition to the tests of ovarian reserve, maternal menopause age could be included in fertility counselling of women.

Our results underline that it is crucial to be cautious when interpreting AMH and AFC in OC users in prediction of the reproductive life span. We advise young women with a very low AMH and very few antral follicles to discontinue their hormonal contraception and repeat the AMH serum test and ultrasound scan after 3–6 months.

The concept of a personalized risk assessment and ovarian reserve screening in fertile women seems inevitable in the near future. The primary practical question is how we should counsel women using hormonal contraception on whether or not they have an increased risk of a shortened reproductive life span. When interpreting the assessment of the reproductive markers in women using OC we should bear in mind, that the 'true values' of AMH and AFC presumably are 20% higher than the measured values and one might question the clinical relevance of such minor differences. When counselling on women's reproductive life span the use of AMH and AFC should not stand alone, but merely act as sub-elements in the overall assessment. In light of the uncertainty regarding ovarian reserve assessment in OC users, avoidance of this group in counselling on reproductive life span could be advocated, but this may exclude almost one-third of the women.

The accuracy of predicting women's reproductive life span is yet to be validated. To conclude, we recommend individualized fertility assessment and counselling to be performed by fertility experts to ensure a valid interpretation of screening results and a reasonable estimate of a woman's reproductive potential.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

All authors contributed to the design of the study. K.B.P. wrote the first and successive drafts of the paper. K.B.P. and J.L.F. carried out the statistical analysis. All authors contributed to the interpretation of results, critically revised the draft for intellectual content and approved the final manuscript.

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Conflict of interest

None declared.

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